Docket No. 13784.105005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Edward James Rozhon et al.		
		Group Art Unit:	1651
Serial No.:	09/712,033		
		Examiner:	Marx, Irene
Filed:	November 14, 2000		
		Confirmation:	9130

For: Enteric Formulations of Proanthocyanidin Polymer Antidiarrheal Compositions

DECLARATION BY AKRAM SABOUNI AND MEI-FONG KING

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, Akram Sabouni, also known informally as Adam Sabouni, a citizen of the United States residing at 300 Winston Drive #721, Cliffside Park, New Jersey 07010, and Mei-Fong King, a citizen of the United States residing at 36795 Spruce Street, Newark, CA 94560, do hereby declare and state that:

1. Akram Sabouni is a co-inventor, along with Edward J. Rozhon, Atul S. Khandwala, Gul P. Balwani, Jody Wai-Han Chan, and David F. Sesin, of the subject matter disclosed and claimed in U.S. Application Serial No. 09/712,033 ("the '033 application"), filed November 14, 2000, which claims priority to U.S. Application Serial No. 08/730,772, filed October 16, 1996, now abandoned.

2. Mei-Fong King was a research technician working at Shaman Pharmaceuticals, Inc., under the direction of Akram Sabouni at the time the invention claimed in the '033 application was made.

3. We understand that the claimed invention relates to methods for treating secretory diarrhea in animals by administering a pharmaceutical composition comprising an aqueous soluble proanthocyanidin polymer composition isolated from a Croton species or a Calophyllum species in which the proanthocyanidin polymer composition is formulated to protect the proanthocyanidin polymer composition from the stomach environment, *e.g.*, by formulation with an enteric coating.

4. Akram Sabouni, along with co-inventors Edward J. Rozhon, Atul S. Khandwala, Gul P. Balwani, Jody Wai-Han Chan, and David F. Sesin, previously executed a Declaration of the Inventors Under 37 C.F.R. § 1.131 (hereinafter "the 131 Declaration"), which we understand was submitted to the United States Patent and Trademark Office in connection with the above-identified application on September 27, 2006, and a copy of which is attached hereto as Exhibit A.

5. A copy of Report No. SP-303-E-074 entitled "Effect of Enteric Coated SP-303 on Intestinal Fluid Accumulation in Cholera Toxin-treated Mice" ("the Report") is Exhibit 2 of the 131 Declaration. The Report summarizes the results of two experiments in which enteric coated beads of SP-303 were orally administered to mice who had been given cholera toxin, resulting in a significant reduction in fluid accumulation compared to controls. The experimental design is detailed on pages 4 and 5 of the Report.

6. We are the authors of the Report. Although the dates were removed from the document, we confirm that we authored the Report prior to August 16, 1996. The experiments described in the report were carried out by Mei-Fong King under the direction of Akram Sabouni. In fact, the Report summarizes data from laboratory notebook nos. 333 and 368 of Mei-Fong King, copies of which were included as Exhibits 3 and 4, respectively, of the 131

Declaration. Although the dates on Exhibits 3 and 4 have been removed, we confirm that these documents are dated prior to August 16, 1996 and that the work reported in these Exhibits was carried out prior to August 16, 1996.

7. We understand that the copy of the Report submitted as Exhibit 2 of the 131 Declaration was not signed or dated, although lines for our signatures appear at the end of the report. After diligent review of our files, we have been unable to locate copies of the Report with original signatures. We also understand that Napo Pharmaceuticals, Inc. has been unable to locate any signed copies of the Report. To confirm our authorship of the Report, we hereby sign and date a copy of the Report. This executed version of the Report is attached hereto as Exhibit B. Although we have provided the actual date that we signed the copy of the Report attached hereto, we confirm that we wrote the Report prior to August 16, 1996.

8. We declare further that all statements made in this Declaration of our own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Akram Sabouni Date

Mei-Fong King 27 Aug 07

Mei-Fong King Date

Exhibit A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Rozhon *et al.*	Confirmation No.:	9130
Serial No.:	09/712,033	Art Unit:	1651
Filed:	November 14, 2000	Examiner:	Irene Marx
For:	ENTERIC FORMULATIONS OF PROANTHOCYANIDIN POLYMER ANTIDIARRHEAL COMPOSITIONS	Attorney Docket No.:	11133-004-999

DECLARATION OF THE INVENTORS UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, Edward J. Rozhon, Atul S. Khandwala, Akram Sabouni, Gul P. Balwani, Jody Wai-Han Chan and David F. Sesin, citizens of the United States of America residing at 523 San Carlos Avenue, El Granada, California, 94018, 300 Winston Drive # 721, Cliffside Park, New Jersey 07010, 3398 Bing Hearst Drive, Suwanne, Georgia 30024, 6 Roberts Drive, Princeton, New Jersey 07010, 28065 Thorup Lane, Hayward, California 94542, and 557 Cambridge Drive, Benicia California 94510, respectively, do hereby declare and state that:

1. We are the inventors of the invention that is disclosed and claimed in the above-identified application, Serial No. 09/712,033 filed November 14, 2000, which claims priority to U.S. Application Serial No. 08/730,772 filed October 16, 1996, now abandoned. The claimed invention relates to methods for treating secretory diarrhea in animals by administering a pharmaceutical composition comprising an aqueous soluble proanthocyanidin polymer composition isolated from a Croton species or a Calophyllum species in which the

proanthocyanidin polymer composition is formulated to protect the proanthocyanidin polymer composition from the stomach environment, *e.g.*, coated with an enteric coating.

2. We are providing this Declaration to demonstrate that we conceived of and reduced to practice at least one embodiment of the claimed invention prior to August 16, 1996, which is the publication date of Davenport *et al.*, Pediatric Pulmonology, S13, Abstract 34.

3. Attached hereto as Exhibit 1 are copies of two pages from a Project Plan authored by co-inventor Edward J. Rozhon. We have reviewed Exhibit 1 and although the dates have been removed from this document, the date of this document is prior to August 16, 1996. Also, we confirm that the invention described in Exhibit 1 and all the acts relied upon in Exhibit 1 were carried out by one or more of us or at the direction of one or more of us in the United States of America prior to August 16, 1996. The pages set forth formulations of SP-303, which is an aqueous soluble proanthocyanidin polymer composition isolated from *Croton lechleri*, that protect SP-303 from acid, such as enteric coating and formulating with buffering or acid reducers.

4. Attached hereto as Exhibit 2 is a copy of Report No. SP-303-E-074 entitled "Effect of Enteric Coated SP-303 on Intestinal Fluid Accumulation in Cholera Toxin-treated Mice" ("the Report") authored by co-inventor Akram (Adam) Sabouni and Mei-Fong King, a laboratory technician acting under the direction of co-inventor Akram (Adam) Sabouni. We have reviewed Exhibit 2 and although the dates have been removed from this document, the date of this document is prior to August 16, 1996. Also, we confirm that the invention described in Exhibit 2 and all the acts relied upon in Exhibit 2 were carried out by one or more of us or at the direction of one or more of us in the United States of America prior to August 16, 1996. The Report summarizes the results of two experiments where enteric coated beads of SP-303 were orally administered to mice who had been given cholera toxin.

The experimental design is detailed on page 4 of 10 and page 5 of 10 of the Report. The mouse model used in the these experiments to measure the effect a test compound, in this instance enteric coated SP-303, has on fluid accumulation in mice given cholera toxin is a well known and art-accepted *in vivo* model of the effect of the test compound on diarrhea in animals.

5. On pages 5 of 10 to 8 of 10 the Report, results of the two experiments are presented and discussed. In Experiment I, group C, which was treated with enteric coated beads of SP-303, showed significantly reduced rations of fluid accumulation by an average of 32% and 29% as compared to control groups A and B, see Table 1 and Figure 1. In Experiment II, a single dose of enteric coated beads of SP-303 significantly reduced cholera toxin-induced fluid accumulation after a seven hour incubation with cholera toxin. Compared to control groups A and C, SP-303 enteric coated beads (group B) significantly reduced the ratios of fluid accumulation by an average of 45% and 38%, respectively, see Table 2 and Figure 2.

6. Attached hereto as Exhibit 3 are copies of notebook pages from laboratory notebook no. 333 of Mei-Fong King, a laboratory technician acting under the direction of co-inventor Akram (Adam) Sabouni. We have reviewed Exhibit 3 and although the dates have been removed from this document, the date of this document is prior to August 16, 1996. Also, we confirm that the invention described in Exhibit 3 and all the acts relied upon in Exhibit 3 were carried out by one or more of us or at the direction of one or more of us in the United States of America prior to August 16, 1996. The notebook pages set forth the raw data obtained from carrying out Experiment I discussed in the Report above.

7. Attached hereto as Exhibit 4 are copies of notebook pages from laboratory notebook no. 3368 of Mei-Fong King, a laboratory technician acting under the direction of co-inventor Akram (Adam) Sabouni. We have reviewed Exhibit 4 and although the dates

have been removed from this document, the date of this document is prior to August 16, 1996. Also, we confirm that the invention described in Exhibit 4 and all the acts relied upon in Exhibit 4 were carried out by one or more of us or at the direction of one or more of us in the United States of America prior to August 16, 1996. The notebook pages set forth the raw data obtained from carrying out Experiment II discussed in the Report above.

8. Exhibits 1 to 4 show that, prior to August 16, 1996, we had conceived and we, or persons acting under our direction, had reduced to practice the claimed methods by treating secretory diarrhea in an animal by orally administering a pharmaceutical composition comprising an enteric coated aqueous soluble proanthocyanidin polymer composition isolated from a Croton species or a Calophyllum species.

9. We declare further that all statements made in this Declaration of our own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:_____ _____

Edward J. Rozhon

Date:_____ _____

Atul S. Khandwala

Date: 9-1-2006 A. Sabouni

Akram Sabouni

Date:_____ _____

Gul P. Balwani

Date:_____ _____

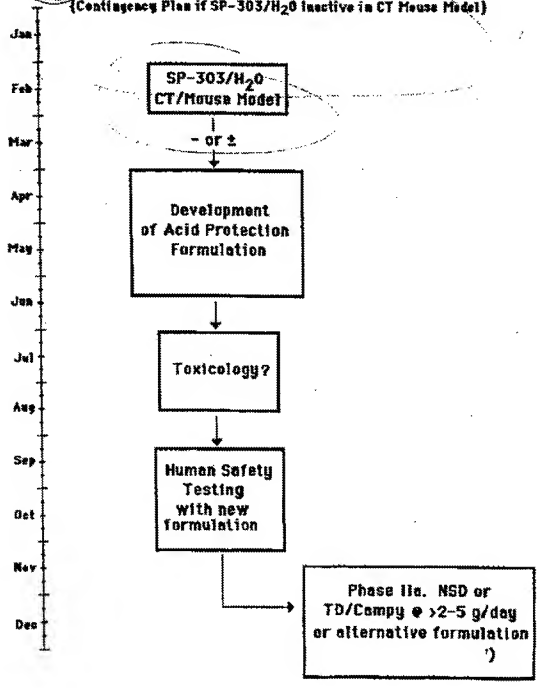
Jody Wai-Han Chan

Date:_____ _____

David F. Sesin

Exhibit 1

PROVIR DEVELOPMENT STRATEGY



E. Rathon

STRATEGY FOR DEVELOPMENT OF PROVIR

(refer to diagrams, pp. 8 to 11)

Introduction: The critical information required to initiate phase IIa studies (page 8) will derive from a study in which SP-303 in H_2O (as opposed to $NaHCO_3$) will be tested in cholera toxin-treated mice. Results showing reduction of intestinal fluid would mean that the IIa studies can begin, whereas, lack of or poor activity activity would require the development of a new formulation to protect against the loss of biological activity due to acid lability of SP-303.

Contingency plan in case SP-303/H_2O is inactive in CT SAM/mouse model (page 9): This scenario would comprise a major set-back for the program in regard to timing, perhaps taking up to a year longer to get to the NDA. The proposal calls for development of a new formulation to protect against acid-caused inactivity of SP-303. The latter may include an enteric-coated capsule, higher dosages of a regular capsule, or giving SP-303 wih an appropriate buffering or acid-reducing system. Subsequent animal tox studies may be required prior to human safety testing. Once the new formulation is developed and proven safe, open label (OL) studies at dosages >2-5 g/day are proposed late in using traveler's diarrhea (TD) and non specific diarrhea (NSD) as described in the paragraph below. Obviously, requirement of a new formulation would impact on the timing and the design of the protocol of the phase I MTD study (see contingency plan below).

Left-hand side flow chart (page 8): Since traveler's diarrhea (TD) and non specific diarrhea (NSD) are different diseases, this strategy assumes that SP-303 will be active against bacterially induced secretory diarrhea (TD) and NSD which is also believed to have a secretory component to its pathophysiology. Our preclinical studies predict activity against enterotoxigenic *E. coli* (ETEC) and other bacteria causing TD, but we have no evidence supporting activity against NSD for which an etiologic agent has never been identified. However, studies by DuPont et al., using antisecretory drugs, have demonstrated activity against both TD and NSD, which suggests the possibility that Provir will have activity against both diarrheal diseases.

Exhibit 2

Abstract

This report summarizes the results of two experiments. The purpose of this study was to determine the effect of enteric-coated SP-303 on fluid accumulation in the intestinal tract of mice treated with cholera toxin (CT). Inhibition of fluid accumulation in this model by a test compound indicates the potential of the compound as an antidiarrheal agent. At initial time (t_0), mice were orally dosed with CT (15 µg per average body weight of ~ 20 g) and anorectally sealed with a cyano-acrylamide ester. Three hours later (t_3 h), a single dose suspension of enteric coated SP-303 (131 mg SP-303/kg) in 0.75% gum guar (vehicle) was administered by oral gavage. Water and control solutions consisting of equivalent concentration of Eudragit and sugar in vehicle were also administered to two control groups. After 6 or 7 hour (t_6 and t_7 h) incubation of CT, mice were sacrificed and the entire murine small intestine tissue from the pylorus to the rectum including cecum was isolated. Fluid accumulation (FA) was measured as the ratio of the mass of accumulated fluid in the small intestine and rectum including cecum versus the mass of the small intestine minus the mass of the fluid. Under the experiment conditions, enteric coated SP-303 was shown to significantly reduce fluid accumulation in the small intestine of sealed adult mice treated with CT. In the second experiment, oral enteric coated SP-303 was capable of reducing the FA ratio by an average of 45% and 38% compared to the mean FA ratio in water controls and Eudragit/sugar/vehicle controls, respectively, at a concentration of 131 mg SP-303/kg.

1. INTRODUCTION

SP-303 is an oligomeric proanthocyanidin isolated from the South American tree *Croton lechleri*. The compound is currently being evaluated in preclinical trials as an antidiarrheal drug candidate. SP-303 previously exhibited activity in the cholera toxin/mouse model when it was administered orally in a solution of 7% $NaHCO_3$ (Reports # SP303-E-069 and SP303-E-070). By contrast, when the compound is dosed in an aqueous vehicle, it is not active. These findings, combined with the known liability of the compound at acidic pH values (in-house unpublished data), suggest that it is necessary to protect SP-303 from the acidic environment of the stomach (pH ~1.5) to obtain activity. Thus, SP-303 was manufactured as enteric coated beads which are designed to resist dissolution at acidic pH. However, once in the neutral to slightly basic pH environment of the small intestine, the enteric coating readily dissolves and releases SP-303. Therefore, the purpose of the studies described in this report were to determine if enteric coated SP-303 would be active in the cholera toxin/mouse model.

Eudragit is an acrylic polymer frequently used in the coating of solid dosage forms. Eudragit films are insoluble below pH 5 and thus resistant to gastric fluid. The Eudragit film dissolves at pH values above 5.5, and thus dissolves in the neutral to weakly alkaline medium of the intestinal

fluid. Enteric coated SP-303 beads were prepared by PMRS (Pharmaceutical Manufacturing Research Services) for testing in the CT mouse model.

2. PURPOSE

The purpose of these experiments was to determine the effect of enteric-coated SP-303 on fluid accumulation in the intestinal tract of mice treated with cholera toxin (CT/mouse model).

3. CHEMICALS AND EQUIPMENT

3.1. Materials

The following materials were obtained from commercial suppliers: cholera toxin (List Biological Lab, lot # CVX-48-3D); cyano-acrylamide ester (Borden Inc., Columbus, OH); animal feeding needles (Popper and Sons, Hyde Park, NY); sodium bicarbonate (ACROS lot # 83559/1); gum guar (Sigma, lot # 94H0195); enteric coated SP-303 (PMRS, lot # R10574); Eudragit L30D (PMRS, lot # R10538); 40-60 mesh sugar spheres (PMRS, lot # R10542).

3.2. Preparation of CT Stock and Dosing Solutions

One ml of HPLC grade water (Mill Q) was added to one List Biological vial containing 1 mg of CT prior to dosing animals. Two different vials were pooled and stored at 4° C. CT dosing solutions were freshly prepared by diluting 240 µl of CT stock solutions with 560 µl of 7% $NaHCO_3$. Final concentration of $NaHCO_3$ was 4.9%. Each mouse received 15 µg of CT in 50 µl volume by oral gavage at initial time (t_0).

3.3. Preparation of SP-303 Enteric Coated Beads

3.3.1. SP-303 layering and Enteric Coating Components

The formulation for enteric coated SP-303 beads contains 17.3% (w/w) of nonpareil seeds (sugar spheres, 46/60 mesh) (Paulaur, lot # 60084060), 64.5% SP-303 (Shaman, lot # PL-097), 1.5% hydroxypropyl methylcellulose (HPMC, Dow Chemical Co., lot # MM9410162E), 0.5% Opadry Clear (Colorcon, lot # S835563), 14.5% Eudragit L30D (Rohm Tech., lot # 1250514132), 1.45% triethyl citrate (Morflex, lot # N5X291), glyceryl monostearate (Rohmn Tech, lot # 502-229), and purified water (USP).

3.3.2. SP-303 layering and Enteric Coating Process

The SP-303 layering coating solution was prepared by adding HPMC and SP-303 to purified water, USP. The SP-303 solution was then mixed well until it was dissolved. The nonpareil seeds were loaded into the product bowl of the fluid bed processor (Niro-Precision Coater). The SP-303/HPMC solution was then layered on the nonpareil seeds by spraying the solution onto the fluidized nonpareil seeds. A target bed temperature was maintained at 30-35° C. SP-303 layering process was continued until all the solution has been applied. Once the SP-303 layering has been completed, a seal coat using Opadry Clear (prepared by mixing the Opadry Clear with Purified Water, USP) was applied. The target bed temperature was maintained at 30-35° C. When the seal coat has been applied, the pellets were discharged and screened through a 1000 µ and 425 µ screens. The layered spheres of which size is larger than 425 µ and smaller than 1000 µ were charged back into fluid bed processor. Meanwhile, the enteric coating solution was prepared by adding triethyl citrate and glyceryl monostearate to water that has been heated to 65° C with continued mixing. This solution was added to the Eudragit L30D-55 while mixing. The resulting enteric coating solution was then sprayed onto the layered spheres in the fluidized bed processor. The bed temperature was maintained at 30-35° C until all enteric coating solution was layered on the beads.

3.4. Preparation of Dosing Solution

To facilitate oral gavage and prevent instantaneous settling of the beads, a thickening agent, gum guar was used. One hundred ml of 0.75% guar gum was prepared and adjusted to pH 2 with 2 ml of 0.5 M HCl. Enteric coated SP-303 beads were suspended in 0.75% gum guar solution. Control solution consisting of equivalent final concentrations of Eudragit and sugar was also prepared in 0.75% gum guar solution.

4. METHODS

The experiments were performed according to Gabriel et al. (Reports # SP303-E-069 and SP303-E-070). 50- to 52-day-old male mice with body masses that ranged from 15.7 to 18.7 g were used. Test animals were wild type C57Bl/6 and were obtained from Charles River Lab. All animals were maintained in metabolism cages with water *ad libidum* for the duration of the experiment. Mice were fasted for 24 hours prior to start of the experiment and were deprived of food during the course of experimentation. Initially (t_0 h), mice were orally dosed with CT (15µg) and anorectally sealed with a cyano-acrylamide ester (Superglue). Three hours later (t_3 h), mice were orally dosed with a suspension of enteric coated SP-303 in gum guar solution. After a 6 (t_6 h) or 7 hour (t_7 h) incubation of CT, mice were sacrificed and the entire murine

small intestine from the pylorus to the rectum including cecum were isolated. Care was taken to avoid tissue rupture and loss of fluid, and the attached mesentery and connective tissue were then removed. The mass of the tissue and the fluid within was determined using an analytical balance. The tissue was then opened longitudinally, the fluid removed, and the tissue was patted dry. Fluid accumulation was measured as ratio of the mass of accumulated fluid in the intestine (small and large including cecum) versus the mass of the intestine minus the mass of the fluid.

5. STATISTICAL ANALYSIS

Statistical comparisons of the fluid accumulation ratio for different treatments were made by analysis of variance using Microsoft Excel (version 5.0). A p-value of $p < 0.05$ was used to determine significance. Duncan's multiple range test was carried out to determine whether statistically significant reductions in CT-induced fluid accumulation ratios occurred in enteric coated SP-303-treated mice compared to animals treated with CT and administered with only H_2O or Eudragit plus sugar in 0.75% gum guar solution.

6. EXPERIMENTAL DESIGN

6.1. Experiment I

Experiment I was carried out on A total of 25 mice (5 mice per each treatment) were used in this study as follows:

Group A: mice were gavaged with CT at t_0 followed by a single dose of H_2O at t_3 and sacrificed at t_6 after CT dosing.

Group B: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of enteric coated SP-303 (131 mg SP-303/kg body weight). The vehicle consisted of a 0.75% gum guar solution. All animals were sacrificed at t_6.

Group C: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of enteric coated SP-303 (131 mg SP-303/kg body weight). The vehicle consisted of a 0.75% gum guar solution. All animals were sacrificed at t_7.

Group D: mice were gavaged with CT at t_0 followed by a single dose of 0.75% gum guar solution at t_3 and sacrificed at t_6 after CT dosing.

Group E: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of

equivalent concentration of Eudragit and sugar (1.33 mg of Eudragit plus 1.046 mg of sugar per kg body weight). The vehicle consisted of acidified 0.75% gum guar solution. All animals were sacrificed at t_6.

6.2. Experiment II

Experiment II was carried out on A total of 24 mice (8 mice per each treatment) were used in this study as follows:

Group A: mice were gavaged with CT at t_0 followed by a single dose of H_2O at t_3 and sacrificed at t_7 after CT dosing.

Group B: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of enteric coated SP-303 (131 mg SP-303/kg body weight). The vehicle consisted of acidified 0.75% gum guar solution. All animals were sacrificed at t_7.

Group C: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of equivalent concentration of Eudragit and sugar (1.33 mg of Eudragit plus 1.046 mg of sugar per kg body weight). The vehicle consisted of acidified 0.75% gum guar solution. All animals were sacrificed at t_7.

7. RESULTS AND DISCUSSION

7.1. Experiment I

Table 1 and Figure 1 show the effect of enteric coated SP-303 beads on inhibition of CT-induced fluid secretion in the sealed adult mouse model. SP-303 significantly ($p<0.05$) reduced CT-induced fluid accumulation following oral single dose administration of 131 mg/kg after a seven hour incubation with cholera toxin (group C). Compared to the control group that was treated with water (group A), and the second control group that received Eudragit plus sugar (group E), group C (SP-303 coated beads in vehicle) significantly reduced the ratios of fluid accumulation by an average of 32% and 29%, respectively. Despite a smaller FA ratio for group C *vs.* group D (gum guar vehicle only), no statistical significance was found between both groups. Furthermore, when the total CT incubation time was only 6 h (not 7 h as for group C), enteric coated SP-303 in vehicle did not reduce fluid accumulation in the intestine (group B). The stomach of several mice in group B was opened at t_6 and visually examined. A significant number of the dosed SP-303 enteric coated beads in gum guar solution was found in the stomach. This might explain the higher FA ratio

found in this group.

As a result of the difficulties encountered in oral gavaging the enteric coated beads, one mouse from group B and C died. Additionally, for unknown reasons, one mouse in group A (water only) died following gavage administration.

Table 1. The effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice

Group	No. of Mice	Treatment	Evaluation Time (h)	Fluid Accumulation* (mg fluid / mg intestine)
A	4	H_2O	6	1.49 ± 0.09 a
B	4	131 mg coated SP-303 in gum guar /kg	6	1.56 ± 0.12 a
C	4	131 mg coated SP-303 in gum guar /kg	7	1.02 ± 0.10 b
D	5	0.75% gum guar	6	1.18 ± 0.08 b
E	5	Eudragit & sugar/ gum guar	6	1.44 ± 0.11 a

* Means not sharing letter in common, differ significantly ($p < 0.05$) by Duncan's Multiple Range Test.

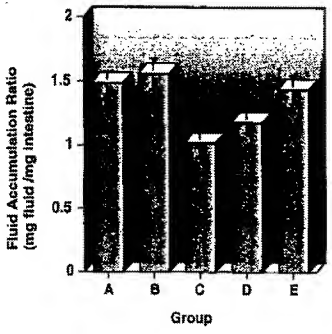


Figure 1. Effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice. Groups A, B, C, D, and E are explained in Table 1 as well as in the text.

6.2. Experiment II

Based on the results obtained in experiment I which indicated the need for longer incubation time to assure complete transfer of the coated beads into the intestine, all animals in experiment II were sacrificed at t_7 after CT dosing. To achieve more reliable results, the number of animals was increased to 8 mice for each group. Table 2 and Figure 2 show the effect of enteric coated SP-303 on CT-induced fluid secretion in the sealed adult mouse model (experiment II). As could be seen, a single dose of 131 mg SP-303/kg significantly ($P<0.05$) reduced CT-induced fluid accumulation after a seven hour incubation with cholera toxin. Compared to the control groups, A and C, SP-303 enteric coated beads (group B) significantly reduced the ratios of fluid accumulation by an average of 45% and 38%, respectively.

No mouse died as a result of oral gavage administration.

Table 2. The effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice

Group	No. of Mice	Treatment	Fluid Accumulation* (mg fluid / mg intestine)
A	8	H_2O	1.28 ± 0.09 a
B	8	131mg SP-303 in gum guar solution/kg	0.71 ± 0.17 b
C	8	Eudragit & sugar/gum guar solution	1.15 ± 0.16 a

* Means not sharing letter in common differ significantly ($p< 0.05$) by Duncan's Multiple Range Test.

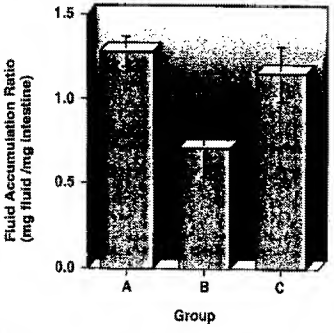


Figure 2. Effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice. Groups A, B, and C are explained in Table 2 as well as in the text.

7. CONCLUSIONS

Under the experimental conditions, enteric coated SP-303 was shown to significantly reduce fluid accumulation in the small intestine of sealed adult mice treated with CT. Based on experiment II, oral enteric coated SP-303 (131 mg SP-303/kg) was capable of reducing the FA ratio by an average of 38%, compared to the mean FA ratio in Eudragit plus sugar controls. These results further support SP-303 as a potential antidiarrheal agent.

8. SIGNATURES

Prepared by: _____ Date_____
Adam Sabouni, Group Leader

Prepared by: _____ Date _____
Mei-Fong King, Scientist

Exhibit 3

NOTEBOOK NO. 333

ISSUED TO Mei-Fong King

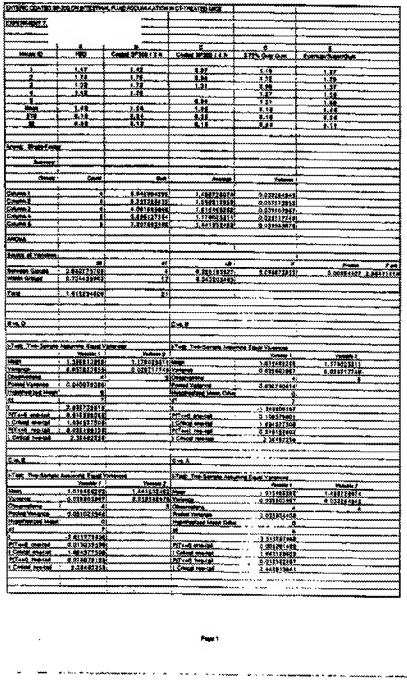
ON

DEPARTMENT PK/Metabolism

RETURNED

microfilmed Pr # 2103

—SCIENTIFIC NOTEBOOK CO.—
2831 LAWRENCE AVE.
P.O. BOX 238
STEVENSVILLE, MI 49127
616-429-8285

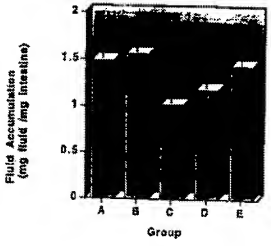


Witnessed & Understood by me, | Date | Invented by | Date |

Recorded by Mei-Fong King

PK/Metabolism

EXPERIMENT 7.
EFFECT OF ENTERIC COATED SP-303 ON FLUID ACCUMULATION IN CHOLERA TOXIN-TREATED MICE



Group	No. of Mice	t0 hr	t3 hr
A	4	CT/$NaHCO_3$	H_2O
B	4	CT/$NaHCO_3$	131mg/kg SP-303
C	4	CT/$NaHCO_3$	*131mg/kg SP-303
D	5	CT/$NaHCO_3$	0.75% Guar Gum
E	5	CT/$NaHCO_3$	Eudragit & Sugar / Gum

* Mice were euthanized at 7 hours after CT dosing.

To Page

Witnessed & Understood by me,	Date	Invented by	Date
		...corded by Mei-Fong Kim	

Exhibit 4

NOTEBOOK NO. 368

ISSUED TO Mei Fang King

ON

DEPARTMENT Pharmacokinetics

RETURNED

microfilmed job # 2103

—SCIENTIFIC NOTEBOOK CO.—
2831 LAWRENCE AVE.
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Project No.
Book No. 368 TITLE Exp. 8 - Effect of enteric coated SP303 on fluid accumulation in CT treated mice

Page No. Notebook #333 page 168

Objective : 1) to determine the effect of enteric coated SP-303 on fluid accumulation in CT- treated mice.
2) to repeat the experiment 7 using lower scale of mice

Experimental : see Notebook #333 page 165 & Anvir Project slide
Experiment date :

Results :

EFFECT OF ENTERIC COATED SP-303 ON INTESTINAL FLUID ACCUMULATION IN CT TREATED MICE

EXPERIMENT

Mouse #	Alive B.W. (g)	Boat tar B.W. (g)	Boat & Mouse (g)	Drug	Boat tar Gut (g)	Boat & Gut & Fluid (g)	Boat & Gut empty (g)	Length of Gut (mm)
A1	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A2	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A3	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A4	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A5	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A6	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A7	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A8	[illegible]	[illegible]	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
MEAN	[illegible]							
STD	[illegible]							
SE	[illegible]							

Mouse #	Alive B.W. (g)	Boat tar B.W. (g)	Boat & Mouse (g)	Drug	Boat tar Gut (g)	Boat & Gut & Fluid (g)	Boat & Gut empty (g)	Length of Gut (mm)
B1	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B2	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B3	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B4	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B5	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B6	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B7	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B8	[illegible]	[illegible]	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
MEAN	[illegible]							
STD	[illegible]							
SE	[illegible]							

Mouse #	Alive B.W. (g)	Boat tar B.W. (g)	Boat & Mouse (g)	Drug	Boat tar Gut (g)	Boat & Gut & Fluid (g)	Boat & Gut empty (g)	Length of Gut (mm)
C1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
MEAN	[illegible]							
STD	[illegible]							
SE	[illegible]							

EFFECT OF ENTERIC COATED SP-303 ON INTESTINAL FLUID ACCUMULATION IN CT TREATED MICE

EXPERIMENT

Mouse #	DOB	Age in Ex (days)	Mouse Wt. (g)	Drug	Gut & Fluid (g)	Gut minus Fluid (g)	Length of Gut (mm)	Fluid (g)
A1	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A2	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A3	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A4	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A5	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A6	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A7	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
A8	[illegible]	32	[illegible]	H2O	[illegible]	[illegible]	[illegible]	[illegible]
MEAN			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
STD			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
SE			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]

Mouse #			Mouse Wt. (g)	Drug	Gut & Fluid (g)	Gut minus Fluid (g)	Length of Gut (mm)	Fluid (g)
B1	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B2	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B3	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B4	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B5	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B6	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B7	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
B8	[illegible]	32	[illegible]	Coated SP-303	[illegible]	[illegible]	[illegible]	[illegible]
MEAN			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
STD			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
SE			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]

Mouse #			Mouse Wt. (g)	Drug	Gut & Fluid (g)	Gut minus Fluid (g)	Length of Gut (mm)	Fluid (g)
C1	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C2	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C3	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C4	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C5	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C6	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C7	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C8	[illegible]	32	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
MEAN			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
STD			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
SE			[illegible]		[illegible]	[illegible]	[illegible]	[illegible]

From Page No. 34

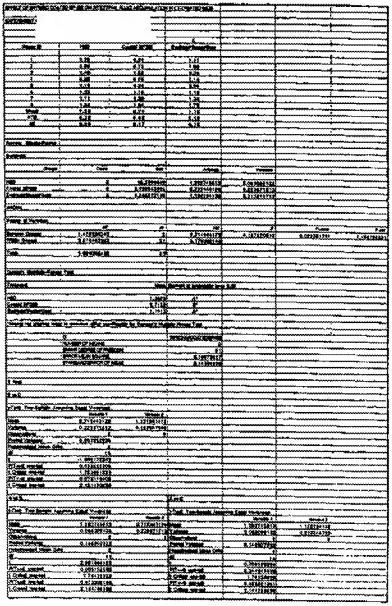
[illegible]

EFFECT OF ENTERIC COATED SP-303 ON INTESTINAL FLUID ACCUMULATION IN CT-TREATED MICE							
EXPERIMENT							
Mouse #	Euthanize Mouse Wt. (g)	Drugs	Gut & Fluid (g)	Gut minus Fluid (g)	Length of Gut (mm)	Fluid (g)	Euthanize Fluid/Gut (g)
A1	17.8	H2O	2.21	0.95	385	1.25	1.26
A2	18.6	H2O	2.34	0.87	385	1.47	1.69
A3	18.1	H2O	1.83	0.65	413	1.18	1.40
A4	17.1	H2O	1.82	0.98	420	0.84	0.86
A5	16.5	H2O	2.08	0.96	350	1.12	1.18
A6	16.7	H2O	2.08	0.82	415	1.26	1.53
A7	18.0	H2O	2.03	0.95	420	1.08	1.11
A8	16.5	H2O	1.83	0.82	380	1.01	1.24
MEAN	17.7		2.04	0.90	404	1.14	1.29
STD	0.8		0.18	0.07	19	0.15	0.26
SE	0.3		0.06	0.03	7	0.07	0.09
Mouse #	Mouse Wt. (g)	Drugs	Gut & Fluid (g)	Gut minus Fluid (g)	Length of Gut (mm)	Fluid (g)	Fluid/Gut (g)
B1	17.0	Coated SP-303	1.28	0.95	420	0.33	0.34
B2	19.5	Coated SP-303	1.90	0.98	406	0.83	0.73
B3	17.9	Coated SP-303	1.94	0.95	460	0.99	1.52
B4	16.5	Coated SP-303	1.21	0.95	370	0.27	0.28
B5	18.9	Coated SP-303	1.37	1.11	458	0.26	0.24
B6	16.2	Coated SP-303	1.88	0.58	418	1.00	1.18
B7	18.1	Coated SP-303	1.47	1.06	438	0.41	0.39
B8	18.4	Coated SP-303	1.53	0.78	385	0.85	1.04
MEAN	17.4		1.61	0.81	406	0.55	0.71
STD	1.0		0.21	0.18	27	0.32	0.48
SE	0.3		0.08	0.05	9	0.11	0.13
Mouse #	Mouse Wt. (g)	Drugs	Gut & Fluid (g)	Gut minus Fluid (g)	Length of Gut (mm)	Fluid (g)	Fluid/Gut (g)
C1	17.9	Eudragit/Sugar/Gum	2.03	0.96	415	1.07	1.11
C2	17.4	Eudragit/Sugar/Gum	1.88	0.85	408	1.01	1.60
C3	18.4	Eudragit/Sugar/Gum	1.03	0.83	400	0.19	0.24
C4	17.3	Eudragit/Sugar/Gum	1.87	0.88	350	1.00	1.14
C5	18.7	Eudragit/Sugar/Gum	1.78	0.81	405	0.86	0.94
C6	18.7	Eudragit/Sugar/Gum	2.07	0.98	458	1.11	1.18
C7	17.2	Eudragit/Sugar/Gum	2.13	0.95	400	1.17	1.24
C8	17.8	Eudragit/Sugar/Gum	2.18	0.78	385	1.40	1.78
MEAN	17.4		1.88	0.88	401	0.88	1.15
STD	0.7		0.38	0.11	18	0.35	0.48
SE	0.2		0.13	0.04	4	0.12	0.15

MFK

To

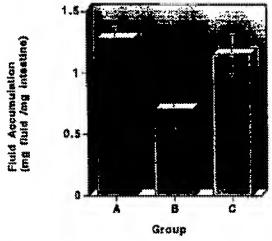
Witnessed & Understood by me,	Date	Invented by	Date
Robert Swezey		Recorded by Mei-Fong Kiy	



Read & Understood by me, | Date | Invented by | Date

PK/Metabolism

EXPERIMENT 8.
EFFECT OF ENTERIC COATED SP-303 ON FLUID ACCUMULATION IN CHOLERA TOXIN-TREATED MICE



Group	No. of Mice	t0 hr	t3 hr	Fluid Accumulation (mg fluid / mg intestine)
A	8	CT/$NaHCO_3$	H_2O	1.28 A*
B	8	CT/$NaHCO_3$	131mg/kg SP-303	0.71 B*
C	8	CT/$NaHCO_3$	Eudragit & Sugar / Gum	1.15 A*

* Means not sharing letter in common differ significantly by Duncan's Multiple RangeTest.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by Mei Fong King

Exhibit B

Abstract

This report summarizes the results of two experiments. The purpose of this study was to determine the effect of enteric-coated SP-303 on fluid accumulation in the intestinal tract of mice treated with cholera toxin (CT). Inhibition of fluid accumulation in this model by a test compound indicates the potential of the compound as an antidiarrheal agent. At initial time (t_0), mice were orally dosed with CT (15 μg per average body weight of ~ 20 g) and anorectally sealed with a cyano-acrylamide ester. Three hours later (t_3 h), a single dose suspension of enteric coated SP-303 (131 mg SP-303/kg) in 0.75% gum guar (vehicle) was administered by oral gavage. Water and control solutions consisting of equivalent concentration of Eudragit and sugar in vehicle were also administered to two control groups. After 6 or 7 hour (t_6 and t_7 h) incubation of CT, mice were sacrificed and the entire murine small intestine tissue from the pylorus to the rectum including cecum was isolated. Fluid accumulation (FA) was measured as the ratio of the mass of accumulated fluid in the small intestine and rectum including cecum versus the mass of the small intestine minus the mass of the fluid. Under the experiment conditions, enteric coated SP-303 was shown to significantly reduce fluid accumulation in the small intestine of sealed adult mice treated with CT. In the second experiment, oral enteric coated SP-303 was capable of reducing the FA ratio by an average of 45% and 38% compared to the mean FA ratio in water controls and Eudragit/sugar/vehicle controls, respectively, at a concentration of 131 mg SP-303/kg.

1. INTRODUCTION

SP-303 is an oligomeric proanthocyanidin isolated from the South American tree *Croton lechleri*. The compound is currently being evaluated in preclinical trials as an antidiarrheal drug candidate. SP-303 previously exhibited activity in the cholera toxin/mouse model when it was administered orally in a solution of 7% $NaHCO_3$ (Reports # SP303-E-069 and SP303-E-070). By contrast, when the compound is dosed in an aqueous vehicle, it is not active. These findings, combined with the known liability of the compound at acidic pH values (in-house unpublished data), suggest that it is necessary to protect SP-303 from the acidic environment of the stomach (pH ~1.5) to obtain activity. Thus, SP-303 was manufactured as enteric coated beads which are designed to resist dissolution at acidic pH. However, once in the neutral to slightly basic pH environment of the small intestine, the enteric coating readily dissolves and releases SP-303. Therefore, the purpose of the studies described in this report were to determine if enteric coated SP-303 would be active in the cholera toxin/mouse model.

Eudragit is an acrylic polymer frequently used in the coating of solid dosage forms. Eudragit films are insoluble below pH 5 and thus resistant to gastric fluid. The Eudragit film dissolves at

pH values above 5.5, and thus dissolves in the neutral to weakly alkaline medium of the intestinal fluid. Enteric coated SP-303 beads were prepared by PMRS (Pharmaceutical Manufacturing Research Services) for testing in the CT mouse model.

2. PURPOSE

The purpose of these experiments was to determine the effect of enteric-coated SP-303 on fluid accumulation in the intestinal tract of mice treated with cholera toxin (CT/mouse model).

3. CHEMICALS AND EQUIPMENT

3.1. Materials

The following materials were obtained from commercial suppliers: cholera toxin (List Biological Lab, lot # CVX-48-3D); cyano-acrylamide ester (Borden Inc., Columbus, OH); animal feeding needles (Popper and Sons, Hyde Park, NY); sodium bicarbonate (ACROS lot # 83559/1); gum guar (Sigma, lot # 94H0195); enteric coated SP-303 (PMRS, lot # R10574); Eudragit L30D (PMRS, lot # R10538); 40-60 mesh sugar spheres (PMRS, lot # R10542).

3.2. Preparation of CT Stock and Dosing Solutions

One ml of HPLC grade water (Mill Q) was added to one List Biological vial containing 1 mg of CT prior to dosing animals. Two different vials were pooled and stored at 4° C. CT dosing solutions were freshly prepared by diluting 240 µl of CT stock solutions with 560 µl of 7% $NaHCO_3$. Final concentration of $NaHCO_3$ was 4.9%. Each mouse received 15 µg of CT in 50 µl volume by oral gavage at initial time (t_0).

3.3. Preparation of SP-303 Enteric Coated Beads

3.3.1. SP-303 layering and Enteric Coating Components

The formulation for enteric coated SP-303 beads contains 17.3% (w/w) of nonpareil seeds (sugar spheres, 46/60 mesh) (Paulaur, lot # 60084060), 64.5% SP-303 (Shaman, lot # PL-097), 1.5% hydroxypropyl methylcellulose (HPMC, Dow Chemical Co., lot # MM9410162E), 0.5% Opadry Clear (Colorcon, lot # S835563), 14.5% Eudragit L30D (Rohm Tech., lot # 1250514132), 1.45% triethyl citrate (Morflex, lot # N5X291), glyceryl monostearate (Rohmn Tech, lot # 502-229), and purified water (USP).

3.3.2. SP-303 layering and Enteric Coating Process

The SP-303 layering coating solution was prepared by adding HPMC and SP-303 to purified water, USP. The SP-303 solution was then mixed well until it was dissolved. The nonpareil seeds were loaded into the product bowl of the fluid bed processor (Niro-Precision Coater). The SP-303/HPMC solution was then layered on the nonpareil seeds by spraying the solution onto the fluidized nonpareil seeds. A target bed temperature was maintained at 30-35° C. SP-303 layering process was continued until all the solution has been applied. Once the SP-303 layering has been completed, a seal coat using Opadry Clear (prepared by mixing the Opadry Clear with Purified Water, USP) was applied. The target bed temperature was maintained at 30-35° C. When the seal coat has been applied, the pellets were discharged and screened through a 1000 μ and 425 μ screens. The layered spheres of which size is larger than 425 μ and smaller than 1000 μ were charged back into fluid bed processor. Meanwhile, the enteric coating solution was prepared by adding triethyl citrate and glyceryl monostearate to water that has been heated to 65° C with continued mixing. This solution was added to the Eudragit L30D-55 while mixing. The resulting enteric coating solution was then sprayed onto the layered spheres in the fluidized bed processor. The bed temperature was maintained at 30-35° C until all enteric coating solution was layered on the beads.

3.4. Preparation of Dosing Solution

To facilitate oral gavage and prevent instantaneous settling of the beads, a thickening agent, gum guar was used. One hundred ml of 0.75% guar gum was prepared and adjusted to pH 2 with 2 ml of 0.5 M HCl. Enteric coated SP-303 beads were suspended in 0.75% gum guar solution. Control solution consisting of equivalent final concentrations of Eudragit and sugar was also prepared in 0.75% gum guar solution.

4. METHODS

The experiments were performed according to Gabriel et al. (Reports # SP303-E-069 and SP303-E-070). 50- to 52-day-old male mice with body masses that ranged from 15.7 to 18.7 g were used. Test animals were wild type C57Bl/6 and were obtained from Charles River Lab. All animals were maintained in metabolism cages with water *ad libidum* for the duration of the experiment. Mice were fasted for 24 hours prior to start of the experiment and were deprived of food during the course of experimentation. Initially (t_0 h), mice were orally dosed with CT

(15μg) and anorectally sealed with a cyano-acrylamide ester (Superglue). Three hours later (t_3 h), mice were orally dosed with a suspension of enteric coated SP-303 in gum guar solution. After a 6 (t_6 h) or 7 hour (t_7 h) incubation of CT, mice were sacrificed and the entire murine small intestine from the pylorus to the rectum including cecum were isolated. Care was taken to avoid tissue rupture and loss of fluid, and the attached mesentery and connective tissue were then removed. The mass of the tissue and the fluid within was determined using an analytical balance. The tissue was then opened longitudinally, the fluid removed, and the tissue was patted dry. Fluid accumulation was measured as ratio of the mass of accumulated fluid in the intestine (small and large including cecum) versus the mass of the intestine minus the mass of the fluid.

5. STATISTICAL ANALYSIS

Statistical comparisons of the fluid accumulation ratio for different treatments were made by analysis of variance using Microsoft Excel (version 5.0). A p-value of $p < 0.05$ was used to determine significance. Duncan's multiple range test was carried out to determine whether statistically significant reductions in CT-induced fluid accumulation ratios occurred in enteric coated SP-303-treated mice compared to animals treated with CT and administered with only H_2O or Eudragit plus sugar in 0.75% gum guar solution.

6. EXPERIMENTAL DESIGN

6.1. Experiment I

Experiment I was carried out on May 10, 1996. A total of 25 mice (5 mice per each treatment) were used in this study as follows:

Group A: mice were gavaged with CT at t_0 followed by a single dose of H_2O at t_3 and sacrificed at t_6 after CT dosing.

Group B: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of enteric coated SP-303 (131 mg SP-303/kg body weight). The vehicle consisted of a 0.75% gum guar solution. All animals were sacrificed at t_6.

Group C: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of enteric coated SP-303 (131 mg SP-303/kg body weight). The vehicle consisted of a 0.75% gum guar solution. All animals were sacrificed at t_7.

Group D: mice were gavaged with CT at t_0 followed by a single dose of 0.75% gum guar solution at t_3 and sacrificed at t_6 after CT dosing.

Group E: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of equivalent concentration of Eudragit and sugar (1.33 mg of Eudragit plus 1.046 mg of sugar per kg body weight). The vehicle consisted of acidified 0.75% gum guar solution. All animals were sacrificed at t_6.

6.2. Experiment II

Experiment II was carried out on May 13, 1996. A total of 24 mice (8 mice per each treatment) were used in this study as follows:

Group A: mice were gavaged with CT at t_0 followed by a single dose of H_2O at t_3 and sacrificed at t_7 after CT dosing.

Group B: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of enteric coated SP-303 (131 mg SP-303/kg body weight). The vehicle consisted of acidified 0.75% gum guar solution. All animals were sacrificed at t_7.

Group C: mice were gavaged with CT at t_0. At t_3, the mice received a single dose of equivalent concentration of Eudragit and sugar (1.33 mg of Eudragit plus 1.046 mg of sugar per kg body weight). The vehicle consisted of acidified 0.75% gum guar solution. All animals were sacrificed at t_7.

7. RESULTS AND DISCUSSION

7.1. Experiment I

Table 1 and Figure 1 show the effect of enteric coated SP-303 beads on inhibition of CT-induced fluid secretion in the sealed adult mouse model. SP-303 significantly ($p<0.05$) reduced CT-induced fluid accumulation following oral single dose administration of 131 mg/kg after a seven hour incubation with cholera toxin (group C). Compared to the control group that was treated with water (group A), and the second control group that received Eudragit plus sugar (group E), group C (SP-303 coated beads in vehicle) significantly reduced the ratios of fluid accumulation by an average of 32% and 29%, respectively. Despite a smaller FA ratio for group C *vs.* group D (gum guar vehicle only), no statistical significance was

found between both groups. Furthermore, when the total CT incubation time was only 6 h (not 7 h as for group C), enteric coated SP-303 in vehicle did not reduce fluid accumulation in the intestine (group B). The stomach of several mice in group B was opened at t_6 and visually examined. A significant number of the dosed SP-303 enteric coated beads in gum guar solution was found in the stomach. This might explain the higher FA ratio found in this group.

As a result of the difficulties encountered in oral gavaging the enteric coated beads, one mouse from group B and C died. Additionally, for unknown reasons, one mouse in group A (water only) died following gavage administration.

Table 1. The effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice

Group	No. of Mice	Treatment	Evaluation Time (h)	Fluid Accumulation* (mg fluid / mg intestine)
A	4	H_2O	6	1.49 ± 0.09 a
B	4	131 mg coated SP-303 in gum guar /kg	6	1.56 ± 0.12 a
C	4	131 mg coated SP-303 in gum guar /kg	7	1.02 ± 0.10 b
D	5	0.75% gum guar	6	1.18 ± 0.08 b
E	5	Eudragit & sugar/ gum guar	6	1.44 ± 0.11 a

* Means not sharing letter in common, differ significantly ($p < 0.05$) by Duncan's Multiple Range Test.

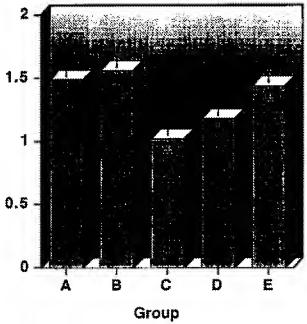


Figure 1. Effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice. Groups A, B, C, D, and E are explained in Table 1 as well as in the text.

6.2. Experiment II

Based on the results obtained in experiment I which indicated the need for longer incubation time to assure complete transfer of the coated beads into the intestine, all animals in experiment II were sacrificed at t_7 after CT dosing. To achieve more reliable results, the number of animals was increased to 8 mice for each group. Table 2 and Figure 2 show the effect of enteric coated SP-303 on CT-induced fluid secretion in the sealed adult mouse model (experiment II). As could be seen, a single dose of 131 mg SP-303/kg significantly ($P<0.05$) reduced CT-induced fluid accumulation after a seven hour incubation with cholera toxin. Compared to the control groups, A and C, SP-303 enteric coated beads (group B) significantly reduced the ratios of fluid accumulation by an average of 45% and 38%, respectively.

No mouse died as a result of oral gavage administration.

Table 2. The effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice

Group	No. of Mice	Treatment	Fluid Accumulation* (mg fluid / mg intestine)
A	8	H_2O	1.28 ± 0.09 a
B	8	131mg SP-303 in gum guar solution/kg	0.71 ± 0.17 b
C	8	Eudragit & sugar/gum guar solution	1.15 ± 0.16 a

* Means not sharing letter in common differ significantly ($p < 0.05$) by Duncan's Multiple Range Test.

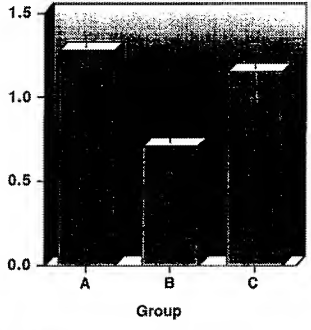


Figure 2. Effect of enteric coated SP-303 beads on intestinal fluid accumulation in CT-treated mice. Groups A, B, and C are explained in Table 2 as well as in the text.

7. CONCLUSIONS

Under the experimental conditions, enteric coated SP-303 was shown to significantly reduce fluid accumulation in the small intestine of sealed adult mice treated with CT. Based on experiment II, oral enteric coated SP-303 (131 mg SP-303/kg) was capable of reducing the FA ratio by an average of 38%, compared to the mean FA ratio in Eudragit plus sugar controls. These results further support SP-303 as a potential antidiarrheal agent.

8. SIGNATURES

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